An evaluation of the *apx*IVA based PCR-REA method for differentiation of *Actinobacillus pleuropneumoniae*

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Abstract

A restriction analysis of PCR (PCR-REA) amplified *apx*IVA gene has been suggested as an alternative method for serotyping of *Actinobacillus pleuropneumoniae* by Jaglic et al. (2004). The current study investigated whether this alternative method could distinguish between the reference strains of serovar 13, 14 and 15 and the value of the method when applied to 47 field isolates representing serovars 1, 2, 3, 5, 7, 8, 9, 12 and 15 as well as non-typable isolates. The reference strains of serovars 13 and 14 had the same sized product after the *apx*IVA PCR, while the product for serovar 15 was of different size compared to all the other serovar reference strains. The *Cfo*I digest profiles of the reference serovar 13 and 14 strains were different from each other and from all other serovars. The *Hpa*II digest profiles of these two serovars were very similar to each other, but both were distinctively different from the other serovar profiles. The *Cfo*I digest profile of serovar 15 strain was very similar to the serovars 3 and 12 strains except for two faint extra bands for serovar 15. The *Hpa*II digest profiles of serovar 12 and 15 reference strains were identical. The PCR-REA method correctly recognized the serovar of 21 of 43 field isolates. It was concluded that the method was a useful additional tool to support, but could not replace, conventional serotyping.

Keywords: *Actinobacillus pleuropneumoniae*; *apx*IVA; PCR-REA; *Cfo*I; *Hpa*II

1. Introduction

Actinobacillus pleuropneumoniae, the causative agent of porcine pleuropneumonia, contributes substantially to economic losses in the swine industry worldwide (Nicolet, 1992). Nielsen et al. (1997) proposed the integration of the serotyping scheme for *A. pleuropneumoniae*, which was previously split into two biovars 1 and 2, which resulted in the recognition of serovars 1 to 14. Serovar 15 was described by Blackall et al. (2002).

Serotyping for *Actinobacillus pleuropneumoniae* is complicated by cross-reactions amongst strains of serovars (Dubreuil et al., 2000). Strong cross-reactions are observed between serovars 1, 9 and 11; 3, 6 and 8 and 4 and 7 (Mittal et al., 1988; Mittal and Bourdon, 1991; Mittal et al., 1993). In a recent study by Kucerova *et al*. (2005), 245 isolates of *A. pleuropneumoniae* were serotyped with 7.8 % displaying a two-way cross-reaction between serovar 9 and 11. The history of serovar 15 is another example of the difficulties with serotyping cross-reactions. The isolates obtained from Australian pigs that are now recognized as serovar 15 were originally assigned to serovar 12 (Blackall et al., 2002).

Jaglic et al. (2004) proposed a restriction enzyme analysis of PCR amplified *apx*IVA gene (PCR-REA) as an alternative method to serotyping for differentiating serovars of *A. pleuropneumoniae*. In their study, Jaglic et al. (2004) examined the reference strains of serovar 1 to 12 as well as field isolates that were cross-reactive with antisera to serovars 1, 9 and 11. In the current study, the work of Jaglic et al. (2004) is extended by examining the reference strains of serovars 13 to 15 and analyzing 40 field isolates (of various serovars) from Australia, 6 from Indonesia and

one from New Zealand to investigate whether the method could be used as an alternative to conventional serotyping of *A. pleuropneumoniae* in Australia.

2. Materials and Methods

2.1 Bacterial strains, media and growth conditions

The reference strains of *A. pleuropneumoniae*, held in the Animal Research Institute culture collection, were (in order of 1 to 15) – 4074, 4226, 1421, M 62, K17, Femo, WF 83, 405, CVJ 13261, 13039, 56153, 1096, N 273, 3906 and HS 143. A total of 47 pig field isolates of *A. pleuropneumoniae* were examined (40 from Australian, six from Indonesia and one from New Zealand). All the field isolates were confirmed as *A. pleuropneumoniae* by phenotypic methods as previously described (Blackall and Pahoff, 1995) or by the PCR described by Gram and Aherns (1998). All the field isolates were serotyped by either the gel diffusion and/or the indirect haemagglutination tests using antisera to the reference strains, listed above, for serovars 1 to 12 and 15 as previously described (Eaves and Blackall, 1988; Blackall and Pahoff, 1995; Blackall et al., 2002). Full details of the field isolates are in Table 1.

All bacteria were grown at 37^0C on BA/SN agar, which consists of BBL™ Blood Agar Base (Becton Dickinson, Sparks MD USA). Just before pouring the media was supplemented with 0.0025% of NADH, 0.0005% of Thiamine HCl, 1% of heat inactivated horse serum and 5% of oleic acid bovine albumin complex which consists of 4.75% bovine serum albumin (fraction V) in normal saline (with the normal saline containing 0.06% oleic acid and 5% 0.05N NaOH).

2.2. PCR-REA Analysis

A 1 µl loopful of *A. pleuropneumoniae* from overnight culture was thoroughly suspended in 100 µl ultrapure water, vortexed and kept on ice for 5 min. The suspension was heated at 98°C for 5 min, followed by cooling on ice for 5 min. After another 5 min at 98°C the solution was centrifuged (2 min at 30230 x *g*) and the supernatant collected and stored at -20°C. This method was slightly altered for both reference and field samples of serovar 7 and 15, where cultures were heated at 98°C for 1 min instead of 5 min.

A 2 µl aliquot of the supernatant was used for PCR analysis. The PCR was performed as previously described by Jaglic et al. (2004) with the following alterations. The HotStar Taq Master Mix Kit (QIAGEN, Hilden, Germany) was used instead of the Taq PCR Master Mix Kit. The cycling parameters of the PCR were changed to 95°C for 15 min, followed by 35 cycles at 94°C for 45 sec, 59°C for 45 sec and 72°C for 6 min. The final elongation step was at 72°C for 10 min. Amplification products were visualised by electrophoresis as described (Jaglic et al. 2004).

The digestion of 20 µl PCR product with 6 U of *Cfo*I or 10 U of *Hpa*II (Roche, Mannheim, Germany) was done for 2 hrs at 37°C. Visualisation was on a 1.5% agarose gel run for 1.5 hrs at 80V.

The molecular weight of the *apx*IVA PCR and the bands generated by the subsequent restriction digests were analysed with BioNumerics software (Applied Maths Inc, Sint-Martens-Latem, Belgium).

3. Results

The reference strains and field isolates of serovar 7 and 15 gave very poor yields of PCR product when the cells were heated at 98°C for 5 min. When the incubation time was reduced to 1 min, much better yields of PCR product were obtained.

Running the *apx*IVA PCR product of the 15 reference strains on a gel produced seven different bands. The reference strains of serovar 2, 5, 7 and 8 produced PCR products of the same molecular size of 3,929 – 3,978 kb. PCR of serovars 1, 3, 12, 13 and 14 gave a PCR product of 3,551 – 3,599 kb. Serovars 6 and 10 yielded PCR products of the same band size of 3,066 and 3,044 kb respectively. Serovars 4, 9, 11 and 15 had unique PCR products (2,877, 2,711, 2,535 and 4,390 kb respectively) that differed from all the other serovar PCR products.

The results of the *Cfo*I digest of the *apx*IVA PCR products of the 15 reference strains are shown in Table 2, displayed according to the band matching results of the BioNumerics program. According to detailed profile analysis all 15 reference strains could be distinguished from each other. However, the simple visual examination of the gel picture highlighted that three pairs of serovars would be difficult to distinguish. These difficult pairs were serovars 5 and 7, 8 and 10 and 9 and 11. The gel profile for serovars 3 and 12 was identical and only the low molecular bands of 68

and 74 bp hold them apart in the BioNumerics profile (Table 2). The profiles for serovars 13 and 14 were distinct from the profiles for the other serovars (Table 2). The profile of serovar 15 was very similar to that of serovars 3 and 12, except that serovar 15 had two faint bands of approximately 400 bp.

The results of the *Hpa*II digest of the *apx*IVA PCR product of the 15 reference strains are shown in Table 3 according to the band matching results of the BioNumerics program. The profile for serovar 5 was very similar to the profile for serovar 7 except for one faint band at 225 bp for serovar 7. The profiles of serovars 8 and 10 were clearly distinguishable from each other. The profiles of serovar 9 and 11 were not clearly distinguishable, especially on visual examination. The profiles of serovars 12 and 15 were nearly identical but could be distinguished from serovar 3. The *Hpa*II profiles for serovars 13 and 14 were very similar, but both were distinctively different from the other serovar profiles. However, if the faint bands around the 2,000 and 1,500 bp were not included, then the profile would be very similar to that of serovar 5 and 10, similar to serovar 6 and similar to serovars 2 and 7, both of which have one extra faint band (Table 3).

The results for the field isolates were not as conclusive as the reference strains (Table 1). A summary is shown in Table 4. Overall, of the 43 isolates that were confidently assigned to a serovar by the traditional serotyping tests, 21 were assigned to the same serovar by the PCR-REA method. A further seven isolates were nearly matched using the PCR-REA method. A near match was defined as meaning that the PCR-REA method suggested more than one serovar with one of those serovars being the same as that given by the traditional methods.

The PCR-REA method performed poorly with serovar 1 – none of the 8 isolates were correctly assigned and only two were a near match. A similar poor association was found with serovar 3 – three isolates were mismatched and one isolate was a near match. In contrast, the PCR REA method performed well with serovars 8 (all seven isolates correctly assigned) and 15 (seven of eight isolates correctly assigned). Of the other serovars represented by four or more isolates, PCR-REA gave near matches with most serovar 12 isolates (three of four) and correct or near matches with serovars 5 and 7.

4. Discussion

Several differences between the study by Jaglic et al. (2004) and the current study were observed. Jaglic et al. (2004) only reported two sizes of the amplicon, 3,000 and 3,529 bp for serovars 1 to 12, while the current study observed 6 different bands for these serovars. Amplicon size differences were between 289 to 465 bp for the reference strains of serovars 2, 5, 7, 8, 9, 10 and 11, despite both this study and Jaglic et al. (2004) using the same reference strains.

The *Cfo*I digest profile for reference serovar 5 was similar to serovar 7 in the current study. Three of the field isolates of serovar 5 gave the same profile as the reference serovar 5 strain, while the other three field serovar 5 isolates gave the profile of reference serovar 6 strain. In contrast Jaglic et al. (2004) reported the profile of serovar 6 strain was similar to that of serovar 7 with the exception of an extra band (~1,500 bp) for serovar 6. Unfortunately, Jaglic et al. (2004) did not use the PCR-REA method on any field isolates of serovars 5 or 6.

Another difference between the studies was the presence of two bands around 200 bp in the *Cfo*I digest profile for reference serovar 2 isolate. The comparison between the studies is based on a visual examination, as Jaglic et a.l (2004) did not report band sizes. It is possible that the difference in the serovar 2 *Cfo*I profile is associated with this reliance on a visual examination.

In the current study, the *Cfo*I profile did not allow for a visual separation of the reference strains for serovars 3 and 12. Jaglic et al. (2004) made no note of this difficulty and simply reported that the two strains were distinct.

This study encountered the same difficulty in differentiating between serovars 9 and 11 as reported by Jaglic et al. (2004). However, the difference in the PCR amplicon size found in this study did allow the separation of the two serovars. The serological cross-reactions between serovars 1, 9 and 11 and the genetic relationship between serovars 9 and 11 are well recognised (Chevallier et al., 1998; Dubreuil et al., 2000). The serological cross-reactions between serovars 1, 9, and 11 are a reflection of similarities in the lipopolysaccharide O-antigen (Jacques, 2004; Perry et al., 2005). Therefore, the major difficulties existing in recognizing serovar 1 field isolates by the PCR-REA method, which assigned most of the isolates to serovar 9 or 11, are not surprising.

The finding of similarities in the REA profiles of the reference strains for serovars 5 and 7 and for 8 and 10 are not seen in serotyping methodologies, whereas serovar 7 cross-reacts with serovar 4 (Perry et al., 2005) and serovar 8 cross-reacts

with serovar 6 (Perry et al., 2005). However, Musser et al. (1987), on the basis of a multi-locus enzyme electrophoresis study, found a close relationship between isolates within serovars 1, 5 and 7 and Chevallier et al. (1998) found a relationship between serovars 8 and 10 by PFGE.

Two of the serovar 5 field isolates were assigned to serovar 6 in the PCR-REA method. Chevallier et al. (1998), using PFGE, found a relationship between serovars 5 and 6.

There is now considerable evidence that the population structure of *A. pleuropneumoniae* is clonal (Musser et al. 1987; Hampson et al. 1993; Chevallier et al. 1998) and that unrelated clones may be serologically identical (Chevallier et al., 1998). This clonal structure may explain while the PCR-REA method can correctly assign the serovar of some field isolates, yet fail for other isolates within the same serovar.

Other studies looking at alternative methods, like outer membrane lipoprotein (*omlA*) PCR typing system in combination with an *apx* (*apx* I, II, and III) typing system and *apx* PCR typing system including primers for all *apx* genes, coud not distinguish between serovars 1, 9 and 11 and 2 and 8 (Gram et al 2000).

In conclusion the PCR-REA method of Jaglic et al. (2004) cannot be used as a substitute for conventional serotyping of *A. pleuropneumoniae*. However, the method does have the potential to be an additional tool to support serotyping, particularly for

serovars 2, 5, 7, 8, 12 and 15. The method might be helpful to shed some light on clonal relationships between samples.

5. Acknowledgment

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Table 1 Comparison of conventional serotyping (by GD or IHA) and serovar assignment by PCR-REA of the *apx*IVA gene using either *Cfo*I or *Hpa*II[a].

Field Isolates[a]	GD /IHA serovar	Serovar based on		
		Size of *apx*IVA product	*Cfo*I digest	*Hpa*II digest
25	1[b]	1, 3, 12, 13 or 14	9	1 or 3
1851	1	1, 3, 12, 13 or 14	11	4, 9 or 11
1893	1	1, 3, 12, 13 or 14	11	1 or 3
2034	1	11	11	4, 9 or 11
2043	1	11	9	4, 9 or 11
2089, 2097, 2099	1	6 or 10	10	14
588, 984	2	2	2	ND[e]
77	3[b]	2, 5, 7 or 8	?[d]	ND
612	3	15	15	ND
721	3	1, 3, 12, 13 or 14	3 or 12	1 or 3
523	3 + 6	?	?	ND
1373, 2230	5	2, 5, 7 or 8	5	ND
1852, 2018	5	6 or 10	6	ND
2214	5	2, 5, 7 or 8	5	?
30	7[b]	2, 5, 7 or 8	7	ND
1861	7	?	5 or 7	?
1891	7	2, 5, 7 or 8	7	ND
2325	7	?	?	ND
1172, 2054, 2074, 2075	8	2, 5, 7 or 8	8	8
1383	8	2, 5, 7 or 8	8	ND
1926	6 + 8	2, 5, 7 or 8	8	ND
1965	8 (6)	2, 5, 7 or 8	8	ND
17	9[b]	2, 5, 7 or 8	?	2, 5 or 10
195, 199	12 (15)	?	3 or 12	1 or 3
872	12	1, 3, 12, 13 or 14	3 or 12	1 or 3
1413	12	6 or 10	?	?
160	15[c]	?	15	ND
173, 451	15	?	15	ND
1937	15	11	4	4, 9 or 11
2235, 2333, 2334, 2335	15	15	15	ND
409	NT	15	15	ND
2038	NT	1, 3, 12, 13 or 14	3 or 12	?
2192	NT	?	7	7
2207	NT	?	4	ND

[a] All isolates were from Australian pigs except that isolates 2054, 2074, 2075, 2089, 2097 and 2099 which were from Indonesian pigs and isolate 2230 which was from a New Zealand pig

[b] These isolates have been confirmed as the indicated serovar by a second laboratory (Boekema et al., 2004)

[c] This isolate has been confirmed as serovar 15 by a second laboratory (Blackall et al. 2002)

[d] ? = no match with any band or profile type seen in the 15 reference strains

[e] ND = not done

Table 2 Molecular weight of bands following *Cfo*I digestion of the *apx*IVA amplification product of the reference strains of *A. pleuropneumoniae*.

Size (kb) of bands following *Cfo*I digestion of *apx*IVA PCR product of the reference strain of the indicated *A. pleuropneumoniae* serovar														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
													2988	
														2504
		2375	2383								2392			
										2275[a]				
								2194[a]						
												1961		
				1865										
	1812													
					1789	1784	1798							
									1723			1742[a]		
1545														
								1520						
										1448				
												1206[a]		
				1046	1055	1053								
	1021[a]													
							879							
875	865	873		870		873		852	863		859			866
										837		847		
455														438
413														388
							376[a]		381			382		
													228	
224	220	224		220	219	224		225		226	223			222[a]
			216											
	172													
												117		
			102		99[a]	102[a]		98	112[a]	110[a]			98[a]	
								75			74	71*	74	
70				70	70	70	70		72	73				71
		68												

[a] refers to weak bands

Table 3 Molecular weight of bands following *Hpa*II digestion of the *apx*IVA amplification product of the reference strains of *A. pleuropneumoniae*.

Size (kb) of bands following *Hpa*II digestion of *apx*IVA PCR product of the reference strain of the indicated *A. pleuropneumoniae* serovar														
1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
												1992[a]	2015[a]	
											1657			1662
1625		1606								1590				
			1555					1563						
	1531[a]						1540							
													1518[a]	
												1424[a]		
	1106				1112	1122								
				1081					1081					
												1035	1054	
890		890					881[a]				878			891
657		658			659	661								
	654			650			643	648		648	656	635	644	653
			638						631					
	451			449	463	460	458		452			450	454	
									406					
399[a]	401			398	390	400	404	394		394		399	391	
		387	382											
						225[a]								
			98											
								77						
71				69	71	72	72		73	72	71			
		68											68	
	62													64

[a] Faint band

Table 4 Summary of outcome of serovar assignment by PCR-REA of the *apx*IVA gene using either *Cfo*I or *Hpa*II.

GD /IHA serovar	Total Number of Isolates	Results of serovar assignment by PCR-REA method		
		Matches with Serotyping	Near Match with Serotyping[a]	Mismatch with Serotyping[b]
1	8	0	2	6
2	2	2	0	0
3	4	0	1	3
5	5	3	0	2
7	4	2	1	1
8	7	7	0	0
9	1	0	0	1
12	4	0	3	1
15	8	7	0	1
Total	43	21	7	15

[a] Near match defined as meaning that the PCR-REA method suggested more than one serovar with one of those serovars matching the serovar identified by GD/IHA testing

[b] Mismatch defined as meaning that the PCR-REA method suggested a serovar or serovars that did not match with the GD/IHA testing or resulted in a pattern not present in the serovar reference strains.